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Biochemical and molecular responses of cyprinids in two Mediterranean lacustrine ecosystems: Opportunities for ecological assessment and biomonitoring

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ABSTRACT

Lacustrine ecosystems have been altered by accelerating pollution, excessive nutrient and organic load, water abstraction, and are susceptible to climate change. Hence, suggesting sensitive and reliable biomarkers for early assessments of their status is of urgent need. In this study, two freshwater commercial fish species, *Cyprinus carpio* (carp) and *Carassius gibelio* (prussian carp) from two lakes (i.e. Koronia and Volvi, Northern Greece) with different anthropogenic pressures were used and a battery of biochemical and molecular biomarkers related to stress response were analyzed in fish gills and liver. In parallel, water physicochemical parameters (T, DO, pH, conductivity, salinity), BOD_5 and nutrient ($N\text{-}NO_3$, $N\text{-}NO_2$, $N\text{-}NH_4$, $P\text{-}PO_4$) concentrations were measured. Results showed that Lake Koronia had higher conductivity and salinity values and $N\text{-}NO_2$ concentrations. Levels of Heat Shock Response (HSR), MAPK phosphorylation, protein carbonylation, lipid peroxidation products, Bax/Bcl-2 ratio, ubiquitination and caspases were increased in gills and liver of both fish species sampled from Lake Koronia in relation to those of Lake Volvi. Likewise, liver lipid content was increased in both fish species sampled from Lake Koronia compared to those sampled from Lake Volvi. The results indicate and reflect the higher environmental degradation that prevails in Lake Koronia ecosystem in comparison to that of Lake Volvi. The fish species studied showed different susceptibility depending on the biomarkers examined. In addition, our results from both examined species provide insight into the mechanisms involved in acclimatization to stressful environments and support the role of the studied biomarkers as sensitive and reliable tools for ecological assessments of lake ecosystems in biomonitoring studies.

1. Introduction

Anthropogenic emissions are released in lake ecosystems and subsequently affect the physicochemical conditions of the water bodies (Søndergaard and Jeppesen, 2007). Aquatic organisms in these ecosystems are likely affected by changes that may occur in their environment. Among them, fish are considered as valuable indicators of aquatic degradation due to anthropogenic pressures. Thus they are of great significance in the ecotoxicology field and they are used extensively in biomonitoring surveys (Chovanec et al., 2003; Naigaga et al., 2011).

Exposure to pollutants can disturb fish cell homeostasis. One of the most important reasons for this disturbance is oxidative stress caused by the increased reactive oxygen species (ROS), which in turn, promotes oxidation of proteins and lipids, and extensive DNA damage. Specifically, exposure to ROS can cause lipid peroxidation (Gutteridge, 1995; Kaloyianni et al., 2009; Taze et al., 2016), as well as an irreversible modification on amino acid aldehyde or ketone groups (carbonylation), which in turn can lead to adhesion of proteins, and inactivation or inhibition of their function (Levine et al., 1990; Costa et al., 2002; Ghezzi and Bonetto, 2003; Mohanty et al., 2010). These mutated and degraded proteins can be subjected to proteolysis via the ubiquitin pathway (Tedesco et al., 2008). Indeed, lipid peroxidation, protein carbonylation and quantification of ubiquitin conjugates have been reported as biomarkers of oxidative stress in various terrestrial and aquatic species including fish (Almroth et al., 2005; Dowling et al.,

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2006; Dailianis et al., 2009; Kaloyianni et al., 2009; Itziou et al., 2011; Volodymyr and Lushchak, 2011; Taze et al., 2016; Sidiropoulou et al., 2018).

Oxidative stress has also been shown to regulate apoptosis, a highly conserved and orchestrated evolutionary process by which cells are physiologically eliminated without inducing inflammation (Edinger and Thompson, 2004). Apoptosis is considered as a main defense mechanism against environmental stresses and plays a key role during environmental toxicity caused by different pollutants and toxicants in fish, other vertebrates and also invertebrates (Ryter et al., 2007). Therefore, apoptosis may be a useful biomarker as well, since it serves as a molecular control mechanism and hence may provide information concerning the impact of the complex water fluctuating environment and xenobiotic pollutants on aquatic organisms (Franco et al., 2009), including fish (Anvari Far et al., 2017).

Moreover, fish and other aquatic organisms can adapt to gradual environmental changes due to an accumulation of a variety of highly conserved proteins in their cells, including the heat shock proteins (HSPs). This group of proteins significantly contributes to the maintenance of cellular homeostasis, protecting the cells against several environmental or physiological factors, e.g. trace metals (Sanders et al., 1991), organic pollutants (Sanders, 1990) or anoxia (Stenslokken et al., 2010). In parallel with these responses to xenobiotics, signaling pathways via the phosphorylation and thus activation of the mitogen activated protein kinases (MAPKs) hold key roles to a variety of physiological functions (Widmann et al., 1999). Numerous transcriptional factors and their regulatory proteins have been recognized as targets of MAPKs (Yang et al., 2003). Therefore, due to their critical role in changes of environmental conditions, these two groups of proteins could also be used as early warning indicators of pollution (e.g. Guven et al., 1994; Triebskorn et al., 1997; Wang et al., 2008).

The present study aimed at assessing the ecotoxicological response of two fish species, *Cyprinus carpio* (carp) and *Carassius gibelio* (Prussian carp) from two lakes which differ in the degree of the anthropogenic pressures they receive (Lakes Koronia and Volvi), by using biochemical and molecular biomarkers related to the oxidative stress status detected in the cells of fish tissues. To date, the information on the toxicity associated with the water bodies of these two lakes is rather limited, mainly due to the presence of microcystins and scum in water, and their occurrence in fish tissues (Lanaras et al., 1989; Moustaka-Gouni et al., 2004; Papadimitriou et al., 2010). To our knowledge, this is the first study that compares the aquatic ecosystems of these two lakes, using a specific battery of biochemical and molecular biomarkers, in an attempt to relate these biomarkers with environmental variables. The hypothesis tested was based on the assumption that differences in lake environments will be seen in the differences in fish responses to the selected biomarkers. Analysis of the response levels of the two studied fish will contribute to further understanding of freshwater ecosystems' health, offering biomarkers for effective early warning system in biomonitoring studies of aquatic environments. Therefore, this study may further contribute to the development of bioindicators intended to estimate ecological risk in lake ecosystems.

2. Materials and methods

2.1. Study area

Lake Koronia (Fig. 1) (surface area of approximately 35 km^2) is a shallow (maximum depth < 3 m), hyper-eutrophic lake (Mitraki et al., 2004). It has suffered from an extreme reduction of its surface area and its water quantity and quality, due to pollutant overloads (mainly untreated municipal wastes and industrial effluents), resulting in the food web collapse of the lake in 1995 and the massive extinction of all fish and waterfowl populations (Grammatikopoulou et al., 1996). Since then, the lake was periodically completely dried up, while in 2005, a restoration plan (funded by the EU Cohesion Fund) was enforced, aiming at the rehabilitation of lake's environmental status. Since 2014, the lake has an almost stable mean depth of approximately 2 m, thus favoring the re-establishment of fish populations (Petriki et al., 2017).

In contrast, Lake Volvi (Fig. 1), the second largest lake of Greece (surface area 68 km^2), is a deep (maximum depth 21 m), eutrophic (Vardaka et al., 2005) lake of tectonic origin. Its water quality is affected by agricultural activities and animal husbandry (Argynaki, 2016) and it is regarded as less impacted by anthropogenic pressures than Lake Koronia, considering the physicochemical water parameters (Vafeiadou et al., 2011). Some water physicochemical parameters for both lakes for the period 2014–2016 are presented in Table 1.

Both lakes constitute a part of the National Park of the wetlands of Lakes Koronia-Volvi and Macedonian Temp, are characterized as site of international importance covered by the Ramsar Convention and the Habitats Directive (92/43/EC; European Commission 1992), and constitute an Important Area (94/24/EC; European Commission 1994) and Special Protected Areas (Directive 2009/147/EC) for birds (Natura 2000 sites GR1220001 and GR1220009).

2.2. Species selection and sample preparation

Specimens of two commercially important fish species, *Cyprinus carpio* (carp) and *Carassius gibelio* (prussian carp) were provided alive by local commercial fishers in November 2016. Species selection was based on their availability and frequency of their presence in commercial catches. Both species are very common in shallow eutrophic lakes, having a vital role in aquatic food chains (Persson et al., 1991; Parkos et al., 2003) and are distributed to the local market for human consumption, thus they have economic significance (Bobori and Economidis, 2006; Cowx, 2015).

Only adult individuals were used. Thereafter, fish specimens of each species (n = 8) were immediately placed in water containing MS-222 to a final concentration of 0.15 g/l to induce anesthesia. Then, fish were dissected and tissue (liver and gill) samples were collected and immediately frozen in liquid nitrogen, transported to the laboratory and maintained at −80 °C until further biochemical analyses. Fish total length averaged 53.6 cm (± 1.52 cm) for carp (ranging from 52 cm to 55 cm) and 21.1 cm (± 1.09 cm) for prussian carp (ranging from 19.5 cm to 22.5 cm) in Lake Koronia. Carps from Lake Volvi had a mean total length of 53.6 cm (± 6.5 cm) ranging between 46 cm and 62 cm, while prussian carps averaged 26.5 cm (± 2.55 cm) ranging from 23.4 cm to 29.1 cm. Fish handling and treatment were in accordance with the local guidelines for treating animals which comply to the Official Journal of the Greek Government No. 106/30 April 2013 on the protection of animals used for scientific purposes.

Moreover, water parameters such as temperature (°C), salinity (ppt), conductivity (μS/cm), dissolved oxygen (mg/l) and pH were measured *in situ* (using a multi-sensor, Aqua Read 2000), while surface water samples were taken for further chemical analyses of nutrients (nitrogen and phosphorous; Merck photometric method) and BOD_5 estimation.

2.3. Molecular and biochemical indicators

The molecular and biochemical indicators investigated in the present study include: lipid peroxidation, protein carbonylation and ubiquitination as oxidative stress indicators; quantification of cell protective proteins HSP60 and HSP90; members of the pathway of apoptosis (Caspases, Bax and Bcl-2) and finally the phosphorylation of members of the MAPK family (p38 MAPK, p44/42 MAPK and JNKs) as signaling molecules in response to stress. Values expressed as means ± SD of 24 determinations for each molecular/biochemical biomarker.

2.3.1. Quantification of lipid peroxidation

The quantification of lipid peroxidation in gills and liver tissues followed the method described by Niehaus and Samuelsson (1968).

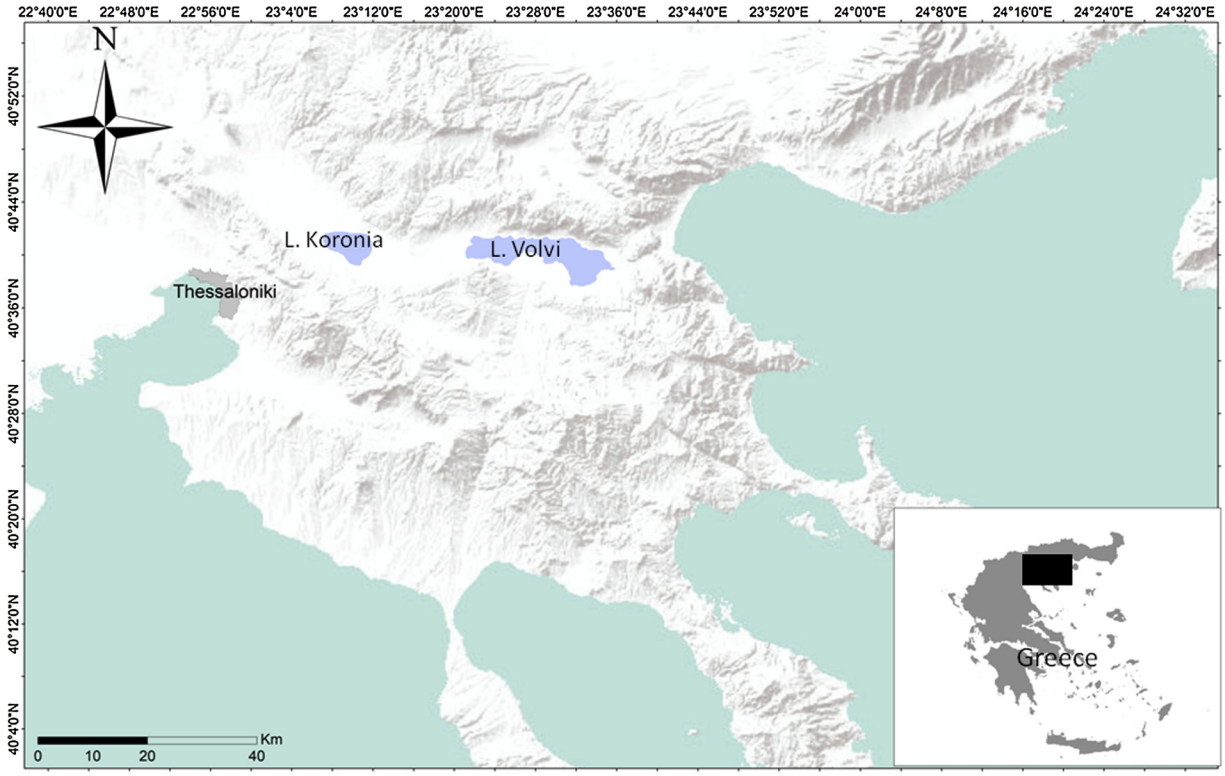

Fig. 1. Schematic map of Lakes Koronia and Volvi (Northern Greece).

Table 1
Physicochemical parameters (mean ± SD) measured in the surface waters of Lakes Koronia and Volvi for the period 2014–2016 (Data provided by the Management Body of Lakes Koronia-Volvi-Chalkidiki), and the sampling period (11/2016) of the present study.

Parameters	Koronia 2014	2015	2016	present	Volvi 2014	2015	2016	present
Temp (°C)	10.45 ± 4.031	17.51 ± 8.809	19.57 ± 7.601	12.6	20.25 ± 6.504	15.57 ± 7.892	15.48 ± 6.048	18.1
DO (mg/l)	7.30 ± 1.556	6.94 ± 3.541	8.90 ± 2.291	10.93	7.30 ± 2.604	11.13 ± 3.326	9.12 ± 0.893	9.69
pH	7.85 ± 0.014	8.25 ± 0.394	8.79 ± 0.381	9.19	± 8.64 ± 0.314	8.79 ± 0.277	8.52 ± 0.159	8.52
COND* (µS/cm)	3427 ± 2055	2638 ± 417	3730 ± 476	4283	1066 ± 47	935 ± 97	998 ± 5	1015
Sal* (ppt)	1.75 ± 1.202	1.36 ± 0.277	1.93 ± 0.231	2.2	0.49 ± 0.020	0.39 ± 0.027	0.4 ± 0.000	0.4
BOD_5 (mg/l)	4.30 ± 1.556	3.4 ± 1.493	2.63 ± 0.416	2.73	3.02 ± 1.976	2.42 ± 0.817	3.23 ± 1.756	1.90
$N\text{-}NO_3$ (mg/l)	0.40	0.446 ± 0.204	0.433 ± 0.561	0.103	0.222 ± 0.211	0.247 ± 0.281	0.448 ± 0.415	0.340
$N\text{-}NH_4$ (mg/l)	0.219	0.145 ± 0.121	0.172 ± 0.147	0.053	0.050 ± 0.031	0.111 ± 0.153	0.092 ± 0.035	0.087
$N\text{-}NO_2$* (mg/l)	0.052	0.071 ± 0.031	0.196 ± 0.258	0.043	0.029 ± 0.006	0.036 ± 0.017	0.049 ± 0.010	0.040
TN (mg/l)	0.671	0.662 ± 0.246	0.801 ± 0.966	0.199	0.302 ± 0.221	0.414 ± 0.424	0.589 ± 0.401	0.467
$P\text{-}PO_4$ (mg/l)	0.280	0.204 ± 0.139	0.133 ± 0.065	0.060	0.084 ± 0.038	0.138 ± 0.166	0.100 ± 0.027	0.130

* represents significant difference between the two lakes, $p < 0.05$.

This experimental procedure is based on the formation of lipid peroxyl radicals and hydroperoxides as the result of the reaction of free radicals or non-radical species with polyunsaturated fatty acids (PUFAs). In this protocol, the results are expressed as nmol MDA per mg protein, since one of the terminal products of lipid peroxidation is MDA (malondialdehyde). The concentration of MDA was detected at 535 nm (ε 1.5 * 105 L mol^{-1} cm^{-1}) (Wills, 1969).

2.3.2. Quantification of protein carbonyl groups with ELISA (Enzyme-linked immunosorbent assay)

Protein carbonylation is estimated after derivatization of protein samples due to their reaction with 2,4-dinitrophenylhydrazine (DNPH) (Buss et al., 1997; Alamdari et al., 2005). The PCC content was quantified according to the standard curve of BSA (bovine serum albumin) measured at 450 nm (Alamdari et al., 2005) using only 5 µg of protein, contrary to the Buss method where 60 µg of protein are required. The results are calculated after extraction of reduced BSA value and are

expressed as nmol carbonyl groups/mg of protein.

2.3.3. Quantification of the molecular chaperones HSP90 and HSP60, members of the MAPK family (phospho p38 MAPK, phospho p44/42 MAPK and phospho JNKs) and the pro-apoptotic Bax and the anti-apoptotic Bcl-2

Liver and gill samples were homogenized in 3 ml/g of cold lysis buffer [20 mmol/l β-glycerophosphate, 50 mmol/l NaF, 2 mmol/l EDTA, 20 mmol/l Hepes, 0.2 mmol/l Na_3VO_4, 10 mmol/l benzamidine, pH 7, 200 μmol/l leupeptin, 10 μmol/l trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane, 5 mmol/l dithiothreitol, 300 μmol/l phenyl methyl sulfonyl fluoride (PMSF), 50 μg/ml pepstatin, 1% v/v Triton X-100], and extracted on ice for 30 min. Samples were centrifuged (10,000 *g*, 10 min, 4 °C) and the supernatants were boiled with 0.33 volumes of SDS/PAGE sample buffer (330 mmol/l Tris − HCl, 13% v/v glycerol, 133 mmol/l DTT, 10% w/v SDS, 0.2% w/v bromophenol blue). Protein concentrations were determined using the BioRad protein assay. Thereafter, equivalent amounts of proteins (100 μg) were separated either on 10% and 0.275% (w/v) acrylamide and bisacrylamide slab gels and transferred electrophoretically onto nitrocellulose membranes (0.45 μm, Schleicher and Schuell, Keene N. H. 03431, USA). Non-specific binding sites on the membranes were blocked with 5% (w/v) non-fat milk in TBST [20 mmol/l Tris − HCl, pH 7.5, 137 mmol/l NaCl, 0.1% (v/v) Tween 20] for 30–45 min at room temperature. Then, the membranes were incubated overnight with polyclonal rabbit anti-bcl2 (Cat. No. 7973, Abcam, Cambridge, MA, USA) and polyclonal rabbit anti-bax (B-9) (Cat. No. 7480, Santa Cruz Biotechnology, Dallas, Texas, USA), polyclonal rabbit anti-HSP90 (Cat. No. 4874, Cell Signaling, Beverly, MA, USA), monoclonal rabbit anti-HSP60 (Cat. No. 12165, Cell Signaling, Beverly, MA, USA), monoclonal rabbit anti-phospho-p38 MAPK (Cat. No. 4511, Cell Signaling, Beverly, MA, USA), monoclonal rabbit anti-phospho-p44/42 MAPK (Cat. No. 4370, Cell Signaling, Beverly, MA, USA) and monoclonal rabbit anti-phospho-SAPK/JNK (Cat. No. 4668, Cell Signaling, Beverly, MA, USA primary antibodies. After washing in TBST (3 periods, 5 min each time), the blots were incubated with horseradish peroxidase-linked secondary antibodies, washed again in TBST (3 periods, 5 min each time), and the bands were detected using enhanced chemiluminescence (Chemicon) with exposure to Fuji Medical X-ray films. Films were quantified by laser-scanning densitometry (GelPro Analyzer Software, Media Cybernetics).

2.3.4. Quantification of ubiquitin conjugates and cleaved caspases conjugates levels

The levels of caspases and ubiquitinated proteins in gills and liver were quantified using a solid-phase immunochemical assay as described by Hofmann and Somero (1996). Samples were diluted to a concentration of 5 μg/ml in a saline solution. Then, 100 μl were loaded in triplicate onto a pre-soaked nitrocellulose membrane (0.45 μm) in a dot blot vacuum apparatus (BioRad) and gravity fed through the membrane. The membranes were blocked, incubated for 1.5 h with a polyclonal anti-ubiquitin rabbit antibody (Cat. No. 3936, Cell Signaling, Beverly, MA, USA) and a monoclonal anti-cleaved caspase rabbit antibody (Cat. No.8698 Cell Signaling, Beverly, MA, USA) diluted 1:2500 and then incubated for 1 h with horseradish peroxidase-linked secondary antibodies washed again in TBST (3 periods, 5 min each time). The blots were developed on Fuji Medical X-ray film using enhanced chemiluminescence (Chemicon). Finally, the blots were quantified (GelPro Analyzer Software, Media Cybernetics).

2.3.5. Quantification of lipid accumulation (content %) in the liver

The livers of the selected fish individuals were removed and kept at −80 °C until the sectioning. Frozen liver slices were placed on positively charged glass plates and were applied on a cryostat Leica CM1850 UV. The sections were stored at −80 °C until staining. Lipids were stained by oil red O procedure as described by Preece (1972). The surface of lipids was quantified using image analysis. The image analysis system consisted of a JVC video camera mounted on an Olympus CX41 light microscope. Lipids quantification was contacted by Image J.

2.4. Statistical analyses

Data were expressed as mean ± standard deviation (± SD). Significant differences ($p < 0.05$) between lakes, species and tissues investigated, regarding protein carbonylation, MDA levels, Bax levels, Bcl-2 levels, ubiquitin conjugate levels and cleaved caspase conjugate levels were assessed by two-way analysis of variance (ANOVA) with species and lakes used as fixed factors. Post-hoc comparisons were performed using the Bonferroni test. Levene's test was applied to the independent samples *t*-test for equality of variances in order to compare the mean values of the physicochemical parameters between the two lakes.

Interrelationships between the molecular biomarkers studied were assessed using the non-parametric Spearman correlation analysis. Since most biomarkers are expressed in arbitrary units, we used the ratios i.e. the value of a biomarker observed in each tissue of each species in Lake Koronia divided by the value of the same biomarker in the same tissue and the same species of Lake Volvi, thus permitting further comparisons between the different tissues.

All statistical analyses were performed using GraphPad Prism 5 (San Diego, CA, USA) and SPSS ver.21 (SPSS, Inc. Chicago, USA) softwares.

3. Results

3.1. Physicochemical parameters

The values of the physicochemical parameters measured during the present study in the surface waters of both lakes were generally within the range of those reported for the previous years (2014–2016) (Table 1). Conductivity, salinity and N-NO_2 exhibited higher mean values in Lake Koronia than in Lake Volvi (*t*-test, $P < 0.007$), while for the rest of the parameters examined no significant differences were observed between the two lakes.

3.2. HSPs

The HSP levels in the two examined tissues of the two fish exposed to different environmental conditions of the two lakes are depicted in Fig. 2. Generally, HSP levels in *C. carpio* were found to be significantly higher in most cases compared to those of *C. gibelio* in both examined lakes. In addition, in both liver and gills, HSP60 (Fig. 2B) and HSP90 (Fig. 2A) levels were higher in both fish species derived from Lake Koronia compared to fish from Lake Volvi.

3.3. MAPKs

Fig. 3 illustrates the phosphorylation levels of MAPKs (p38 MAPK, p44/42 MAPK and JNKs) in gills and liver of the two species examined in both lakes. MAPKs' phosphorylation exhibits similar profiles as those of HSPs in fish species sampled from both lakes. MAPK phosphorylation levels were higher in *C. carpio* compared to *C. gibelio*. In addition, significantly higher levels of MAPKs were measured in tissues of fish sampled from Lake Koronia in relation to those of Lake Volvi. All the levels of MAPKs studied showed significant differences between the fish species examined, with the exception of phospho p38 MAPK.

3.4. Bax/Bcl-2 ratio, ubiquitin conjugates and caspases

In Fig. 4, apoptotic markers such as the Bax/Bcl-2 ratio, caspases and ubiquitin conjugates are illustrated in both examined fish liver and gill samples from lakes Koronia and Volvi. In most cases, all the biomarkers from both fish species sampled from Koronia exhibited higher

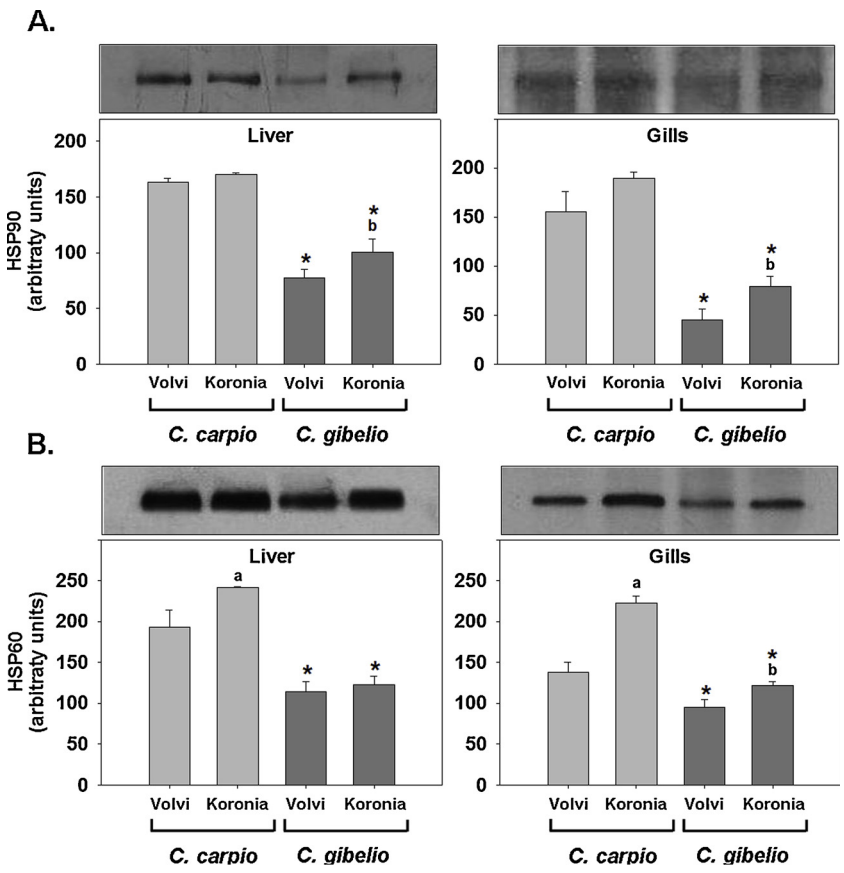

Fig. 2. HSP90 (A) and HSP60 (B) levels in the liver and gills of *Cyprinus carpio* and *Carassius gibelio* in lakes Koronia and Volvi. Values represent mean ± SD of 24 determinations; small letter denotes significant difference between the two examined lakes, while asterisk (*) represents significant difference between the two examined species of the same lake, $P < 0.05$.

values than those sampled from Lake Volvi. Specifically, Bax/Bcl-2 ratio (Fig. 4A) was found to be higher in both examined species and in both fish tissues from Lake Koronia compared to those of Lake Volvi. In addition, this ratio was statistically higher in *C. gibelio* compared to that of *C. carpio*. Caspase levels (Fig. 4B) followed a similar profile, exhibiting higher levels in Lake Koronia in both examined species and tissues.

Concerning the ubiquitin conjugate levels (Fig. 4C), while in the liver of both examined fish species the levels were found to be statistically higher in samples derived from Lake Koronia in relation to those from Lake Volvi, most interestingly, in the gills of both fish species a different response was observed. Specifically, ubiquitin conjugate levels estimated for gill samples of *C. gibelio* from Lake Koronia were lower compared to those for gills of fish sampled from Lake Volvi.

3.5. Protein carbonyl content, lipid peroxidation and lipid liver content

The effect of different lake water status on the integrity of proteins and lipids in the gills and liver of the two species examined is depicted in Fig. 5. Both protein carbonylation (Fig. 5A) and lipid peroxidation (Fig. 5B) exhibited significantly higher levels in tissues of fish sampled from Lake Koronia compared to those from Lake Volvi. In addition, no statistically significant differences were observed between the two species examined. However, there is evidence of a tendency regarding *C. carpio* gills to exhibit higher values in relation to *C. gibelio*.

When lipid content in liver (Fig. 5C) was considered, both species exhibited higher but not statistically significant lipid content in livers of fish derived from Lake Koronia in relation to those from Lake Volvi. However, significant higher values of lipid content were detected in *C. gibelio* compared to *C. carpro.*

3.6. Biomarker interrelationships

The examined interrelationships between the values of molecular biomarkers examined in the tissues of both fish species are presented in Table 2. It is evident that significant interrelations (Spearman's rho; $P < 0.05$) exist, mostly between HSP90 and Bax/Bcl-2 and ubiquitin, HSP60 and p38 MAPK phosphorylation, p44/42 MAPK and JNKs phosphorylation and finally JNKs phosphorylation and (lipid peroxidation) MDA (Table 2).

4. Discussion

Molecular biomarkers in fish reflecting a biological response to physical or chemical alterations caused by anthropogenic pressures that impact aquatic ecosystems, have been used to assess the environmental health (Corsi et al., 2003; Moore et al., 2004; Hook et al., 2014) and have been extensively discussed in relation to their applicability in environmental risk assessment (Beliaeff and Burgeot, 2002; Van der Oost et al., 2003; Hutchinson et al., 2006). In the present study, several biochemical and molecular biomarkers were estimated in two tissues (gills and liver) of two common freshwater cyprinids, the prussian carp and the common carp, originated from two different freshwater ecosystems, lakes Koronia and Volvi. The responses of the two fish species were compared to each other in relation to the parameters tested.

Extant pollutants in aquatic environments, such as transition metals, polycyclic aromatic hydrocarbons, organochlorine and organophosphate pesticides and other xenobiotics, as well as changes in environmental parameters, such as temperature, oxygen levels and salinity (Lushchak, 2011; Hu et al., 2014; Taze et al., 2016) may cause oxidative stress to animals, which result in damaging of all components of the cells, including proteins, lipids, and DNA. Oxidation of proteins and lipids represents an indication of oxidative stress induction and is reflected by enhancement of protein carbonyls and MDA levels.

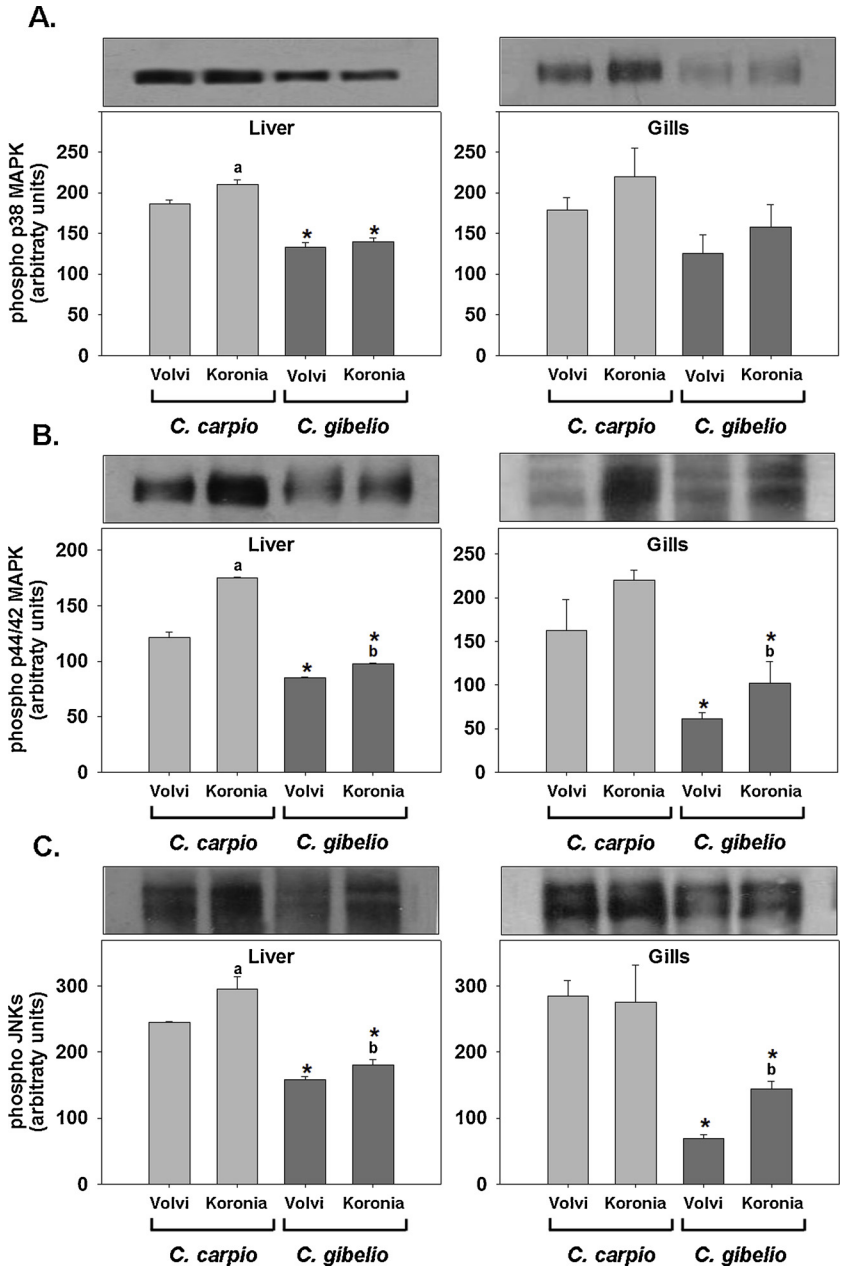

Fig. 3. Phosphorylation levels of p38 MAPK (A), p44/42 MAPK (B) and JNKs (C) in the liver and gills of *Cyprinus carpio* and *Carassius gibelio* in lakes Koronia and Volvi. Values represent mean ± SD of 24 determinations; small letter denotes significant difference between the two examined lakes, while asterisk (*) represents significant difference between the two examined species of the same lake, $P < 0.05$.

Increased MDA levels were observed in fish derived from polluted areas in comparison to those from clean freshwater areas (Radi and Matkovics, 1988; Gül et al., 2004; Karadog et al., 2014; Hermenean et al., 2015). Our results showed elevated oxidative stress levels due to increased oxidation of proteins (protein carbonyls) and lipids (MDA) in gills and liver of both fish species sampled from Lake Koronia in relation to those from Lake Volvi, which denotes increased oxidative stress in fish originated from Koronia. The latter may be attributed to the higher organic content demonstrated by the BOD and nutrient values in water of Lake Koronia in relation to Lake Volvi. Recently, Shuangyao et al. (2018) found that fluctuations of pH resulted in changes of oxidative stress biomarkers, including MDA in juvenile turbot *Scophthalmus maximus*. Furthermore, water temperature influences the antioxidant defense system and oxidative stress biomarkers including oxidative damage to lipids in tissues of the Antarctic fish *Notothenia coriiceps* and *Notothenia rossii* (Klein et al., 2017). Moreover, the concentrations of microcystins determined in scum of both lakes exhibited higher values in Lake Koronia in relation to Lake Volvi (Papadimitriou et al., 2010). The intense human activities in Koronia's basin (Alexandridis et al., 2007) and the pollutants transferred by inflow rivers (Giouri et al., 2010) have resulted in the deterioration of its water status in relation to that of Lake Volvi. Previous studies have proven the assay of measuring protein carbonyls to be a sensitive indicator of protein oxidation (Dalle-Donne et al., 2003) on the mussel *Mytillus galloprovincialis* (Kaloyianni et al., 2009; Patetsini et al., 2013; Koutsogiannaki et al., 2014; Taze et al., 2016) and on the snail *Eobania vermiculata* (Itziou and Dimitriadis, 2011; Sidiropoulou et al., 2018). The present study confirms the sensitivity of this method and encourages its application as a biomarker in *Cyprinus carpio* and *Carassius gibelio*. The advantage of this assay in comparison to the measurement of other oxidation products is the relative early formation and the relative stability of carbonylated proteins. Therefore, MDA and protein

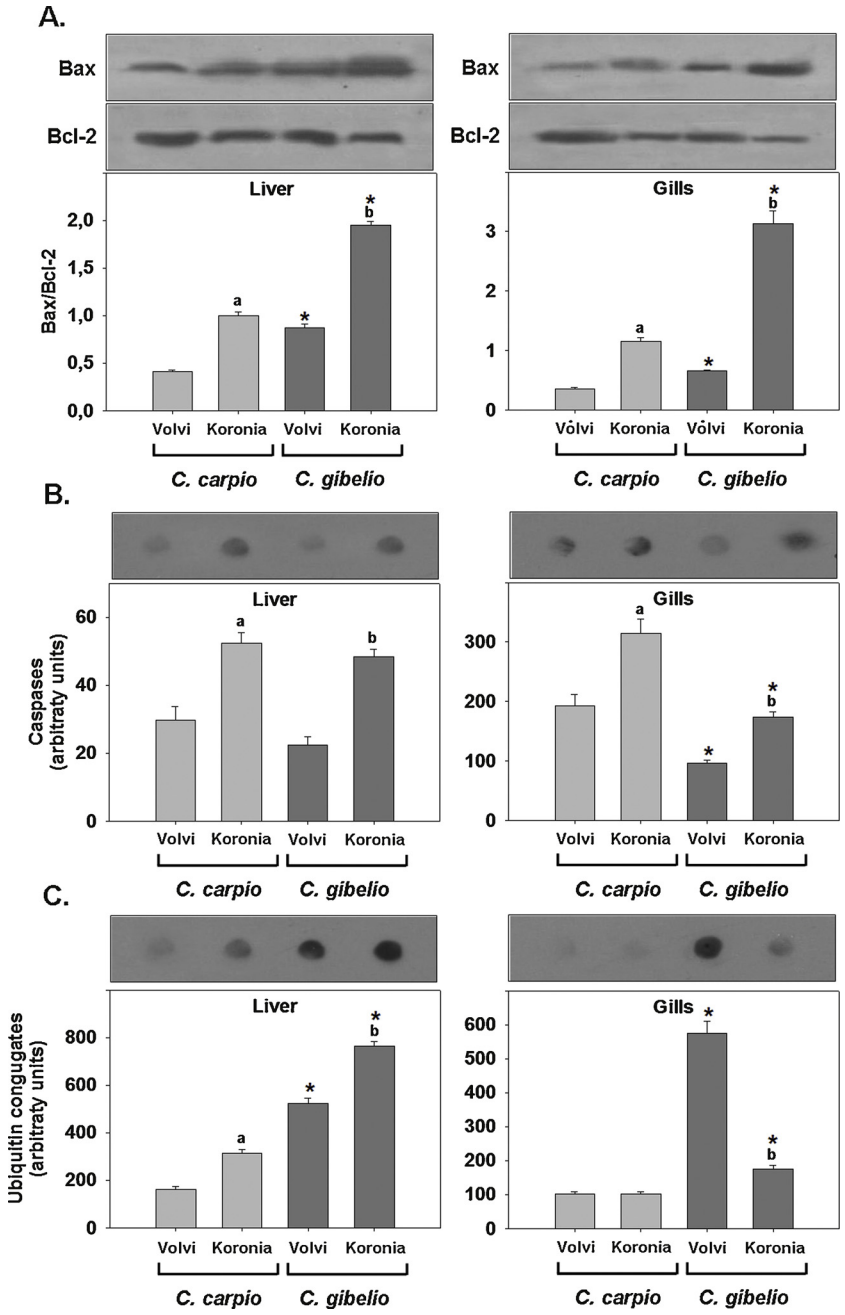

Fig. 4. Bax/Bcl-2 ratio (A), caspase levels (B) and ubiquitin conjugates (C) in the liver and gills of *Cyprinus carpio* and *Carassius gibelio* in lakes Koronia and Volvi. Values represent mean ± SD of 24 determinations; small letter denotes significant difference between the two examined lakes, while asterisk (*) represents significant difference between the two examined species of the same lake, $p < 0.05$.

carbonyls could be suggested as potent and sensitive biomarkers in lake environments.

The mutated or post-translationally damaged proteins that occur after carbonylation (Davies et al., 2001) follow the ubiquitin pathway, along which proteins are degraded. Our results showed that - in accordance with the increased macromolecule oxidation detected in fish tissues sampled from Lake Koronia in relation to those sampled from Lake Volvi - elevated ubiquitin conjugates were also measured in liver of both fish examined. Elevated ubiquitin conjugates observed in animal tissues are commonly reported after exposure to oxidative substances, seasonal fluctuations, hypoxia and hyperoxia events (Hofmann and Somero, 1995, 1996; Joyner-Matos et al., 2006; McDonagh and Sheehan, 2006, 2008) and nanoparticle concentrations (Taze et al., 2016; Sidiropoulou et al., 2018).

The ubiquitin pathway is also involved in apoptosis and antioxidant defense (Ciechanover, 1998) and the increased Bax/Bcl-2 ratio is also related to apoptotic events. Our results showed that caspase levels as well as Bax/Bcl-2 ratio were significantly increased in both tissues of both fish species derived from Lake Koronia in relation to those from Lake Volvi, indicating a more impacted environment in the first one. In accordance with our results, Jiao et al. (2018) reported oxidative stress and apoptosis in common carp gills after chlorpyrifos exposure, concluding that the toxic effects of water environmental pollutants influence the immune function and structural integrity in fish gills. In agreement with the latter, several studies conducted on aquatic animals support the relation of environmental pollutants with apoptosis (Cao et al., 2013; Yavaşoğlu et al., 2016; Altun et al., 2017; Zeeshan et al., 2017).

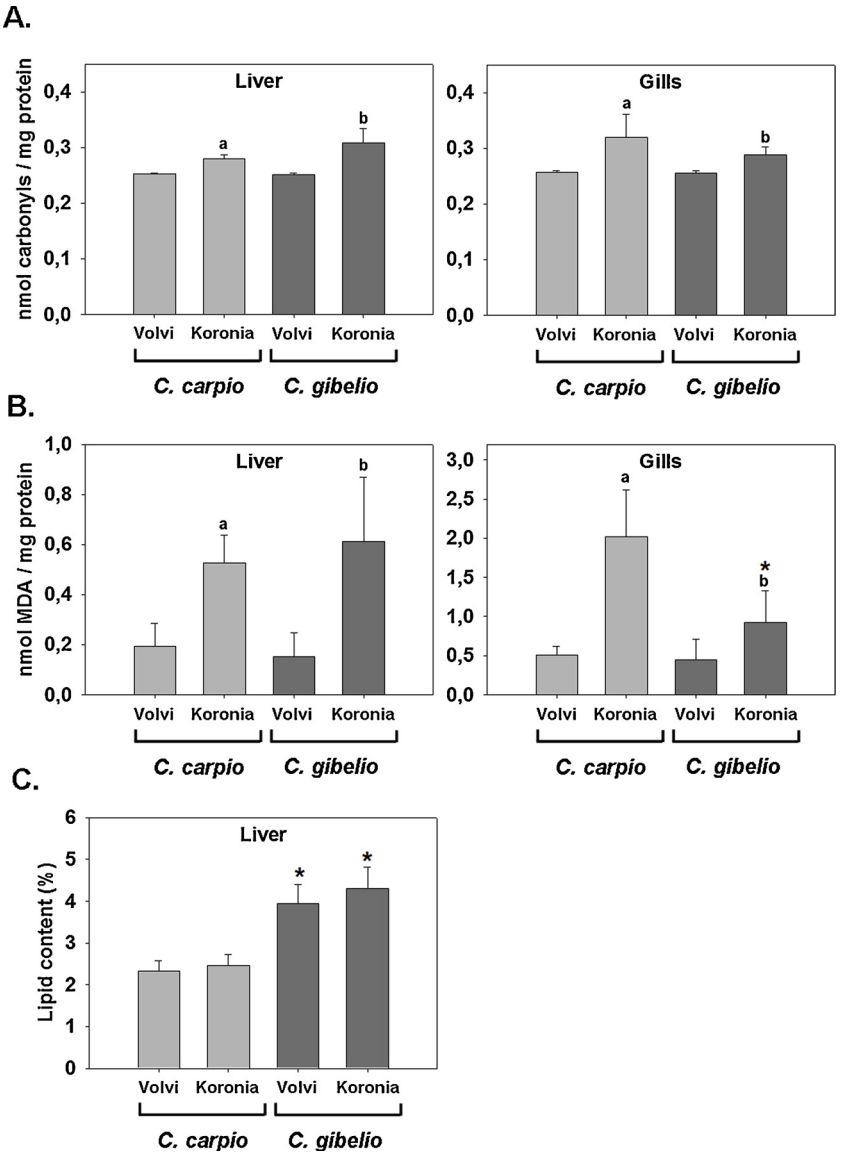

Fig. 5. Protein carbonylation (A) and lipid peroxidation (B) levels in the liver and gills of *Cyprinus carpio* and *Carassius gibelio* in lakes Koronia and Volvi. Lipid content (%) levels (C) in the liver of *Cyprinus carpio* and *Carassius gibelio* in lakes Koronia and Volvi. Values represent mean ± SD of 24 determinations; small letter denotes significant difference between the two examined lakes, while asterisk (*) represents significant difference between the two species examined of the same lake, $P < 0.05$.

Table 2
Spearman's rho correlation of molecular biomarkers. With bold italics: correlation is significant at the 0.01 level, bold: correlation is significant at the 0.05 level.

	HSP90	HSP60	p38 MAPK	p44/42 MAPK	JNKs	Bax/Bcl2	Caspases	Ubiquitin	Carbonyl	MDA
HSP90	1									
HSP60	−0.112	1								
p38 MAPK	0.364	***0.734***	1							
p44/42 MAPK	−0.133	0.266	0.427	1						
JNKs	0.469	0.084	0.406	**0.580**	1					
Bax/Bcl2	**0.580**	−0.559	−0.133	0.084	0.517	1				
Caspases	−0.147	0.573	0.531	0.357	−0.175	−0.413	1			
Ubiquitin	***−0.762***	−0.280	−0.559	−0.224	−0.448	−0.427	−0.308	1		
Carbonyl	0.175	0.063	0.042	−0.007	−0.245	−0.245	0.112	−0.350	1	
MDA	−0.357	0.042	−0.301	−0.441	***−0.853***	−0.371	0.371	0.105	0.357	1

The MAPK signaling pathway plays a vital role in stress responses of animals. This group of proteins plays a key role in a variety of physiological functions and could be used as an early indicator of pollution (Guven et al., 1994; Triebskorn et al., 1997; Wang et al., 2008). We observed increased phosphorylation levels of p38 MAPK, p44/42 MAPK and JNKs in both fish species and tissues derived from Lake Koronia in relation to those sampled from Lake Volvi. Similarly, according to Peng et al. (2015), MAPKs (including p38, JNK, and p44/42) were also significantly increased after Cd^{2+} exposure. On the other hand, phosphorylation of p44/42, p38, and JNK MAPKs triggers activation of

caspase 8 and 3 after cadmium exposure in human osteoblasts (Brama et al., 2012). Therefore, it seems that these molecules (MAPKs and caspases) at certain conditions are interrelated in the cell function. Our results showed high correlation of the apoptotic ratio Bax/Bcl2 with HSP90, of HSP90 with ubiquitin, as well as of JNK with MDA, denoting the close relation of these molecules in the cells of the fish tissues examined.

Elevated levels of HSPs are reported after exposure to several pollutants such as heavy metals (Sanders et al., 1991; Agell et al., 2004), organic pollutants (Sanders, 1990) and in anoxia (Stensløkken et al., 2010). In our study, elevated levels of HSP60 and HSP90 in the examined fish tissues indicate that the animals face conditions that urge them to activate their defense mechanisms. The elevated levels of HSP60 observed in the current study, were correlated with the detected levels of p38 MAPK, thus confirming the close relation of these proteins in tissues of fish suffering polluted environments. Similarly, in the brain of the common carp, a significant increase was observed in the HSP70, HSP60 and HSP90 levels after tributyltin exposure (Li et al., 2016). In addition, Kim et al. (2014) reported that the expression of *hsp20* and *hsp70* genes was significantly altered after exposure to metals, indicating that these genes could be used as a sensitive molecular biomarker for aquatic monitoring of metal pollution.

Between the two examined fish tissues, the profiles of HSP60 and HSP90 and p38MAPK, p44/42 MAPK and JNK phosphorylation were similar in both fish species. Similar profiles between the two studied tissues were also observed when protein carbonyl, caspases and the ratio Bax/Bcl 2 were estimated. The only exception in the response of the two examined tissues was MDA content, that showed elevated values (0.5–2 nmoles MDA/mg protein) in gills of both fish, in relation to liver (0.1-0.6 nmoles MDA/mg protein). Therefore, in relation to MDA, it seems that gill cells are more susceptible to environmental changes than liver cells. In accordance with our results, the gills of the fish *Catla catla* were reported as the most sensitive organ to methyl parathion toxicity compared to liver and plasma (Abhijith et al., 2016). On the other hand, according to Hermenean et al. (2015) and Paruruckumani et al. (2015) liver shows a greater degree of adaptability to counteract ROS compared to kidney in *Leuciscus cephalus* and in copper-treated *L. calcarifer*, respectively.

The interspecies comparisons revealed that when MDA was measured in gills, common carp responded more intensely than prussian carp in both lakes. On the other hand, *C. gibelio* seems to be more easily affected than *C. carpio,* when the ratio Bax/Bcl-2 and lipid content are considered. Therefore, depending on the biomarker examined, either one or the other fish species is more susceptible to environmental stressors.

Among other organs, liver is most related with the detoxification and biotransformation process and also its location, function and blood supply makes this organ more susceptible to contaminants in the water (Rodrigues and Fanta, 1998; Van der Oost et al., 2003). Therefore, evaluation of histological changes in the fish liver is appropriate and accurate approach in order to estimate the effects of xenobiotic compounds in both field and experimental studies. Our results showed that the lipid content expressed a tendency of higher accumulation in the liver in both fish derived from Lake Koronia in relation to those from Lake Volvi. Probably, the apoptosis observed in liver cells had an important causative role in the process of pollutant-induced histological changes in liver. Accordingly, histological changes were observed in liver of fish species originated from polluted wetland environments (Abdel-Moneim et al., 2012; Bernabò et al., 2014). Similarly, van Dyk et al. (2012) and Paruruckumani et al. (2015) suggested that the liver histopathology of sharp-tooth catfish *Clarias gariepinus* and that of the Asian sea bass *Lates calcarifer*, respectively, could be a sensitive biomarker of freshwater aquatic pollution.

Generally, in most cases, fish originated from Lake Koronia presented higher levels of the studied biomarkers in relation to those from Lake Volvi, indicating that the aquatic ecosystem of Koronia is probably more severely affected by anthropogenic pressures in relation to the Volvi ecosystem. The latter is also consistent with the values of the physicochemical parameters measured in the water of the lakes. The present study was conducted during autumn, and the environmental conditions could lead to differences when compared to other seasons (Pavlović et al., 2018). In addition, climatic conditions, the degradation stage of waste, the type of wastes could also alter the pollutants' potency (Tsarpali et al., 2012; Toufexi et al., 2013), causing more divergences in studies' comparisons.

In conclusion, the results of the current study suggest the use of cyprinids as suitable model organisms for the risk assessment of aquatic ecosystems. In addition, our results provide a better understanding of the molecules' contribution to the induction of oxidative stress, as well as to the mechanisms that are induced in the organism to combat pollutants in aquatic ecosystems. HSP expression, MAPK phosphorylation, caspase and ubiquitin conjugate levels, Bax/Bcl-2 ratio and protein carbonyls, lipid peroxidation products, as well as liver histological modifications observed in the fish tissues, support the role of these biomarkers as biochemical and molecular indicators in response to environmental pollution

Authors' contribution

MK and DB designed the study, MK mainly contributed to the writing of the manuscript. Parts of the work were done in the frame of the Diploma theses of TT and PS supervised by DB and EA and of IN supervised by MK and DB with assistance by KF. AD conducted and analyzed the lipid liver content experiments. IN, PS, TT and AD had an equal contribution in performing most of the analyses and DB run the statistical analyses. KF, DB and EA assisted the analyses and contributed to the writing of the manuscript.

Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Declarations of interest

None.

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